The Oncologist®
Gastrointestinal Cancer

Concordance of Predictive Markers for EGFR Inhibitors in Primary Tumors and Metastases in Colorectal Cancer: A Review

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Key Words. Colorectal cancer • Concordance • EGFR inhibitors • *KRAS* • Monoclonal antibodies

Disclosures: Jara M. Baas: None; **Lisanne L. Krens:** None; **Henk-Jan Guchelaar:** *Research funding/contracted research:* Amgen Inc, Merck BV; **Hans Morreau:** None; **Hans Gelderblom:** *Research funding/contracted research:* Amgen Inc, Merck BV.
The content of this article has been reviewed by independent peer reviewers to ensure that it is balanced, objective, and free from commercial bias. No financial relationships relevant to the content of this article have been disclosed by the independent peer reviewers.

ABSTRACT

Background. **Currently, only Kirsten rat sarcoma 2 viral oncogene homolog (*KRAS*) mutational status is used as a decisional marker for epidermal growth factor receptor (EGFR) inhibitor therapy in colorectal cancer (CRC) patients. Concordance of *KRAS* status between primary tumors and metastases has always been considered to be close to perfect; however, cases of discordance have been reported. The actual rate of concordance of *KRAS* status remains unclear, as is the same for v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), phosphatidylinositol 3-kinase CA subunit (*PIK3CA*), and loss of phosphatase and tensin homologue deleted on chromosome ten (*PTEN*). Therefore, it is unknown whether it is necessary to perform mutational analysis on metastases instead of on (or in addition to) primary tumors.**

Design. **A systematic literature search was conducted to collect all studies testing concordance of *KRAS* in CRC, and also of *BRAF*, *PIK3CA*, and loss of *PTEN*.**

Results. **Twenty-one studies have reported concordance of *KRAS*, with an overall concordance rate of 93% (range, 76%–100%). Overall concordance rates of studies testing concordance of *BRAF* status and loss of *PTEN* were 98% and 68%, respectively. Three studies reported concordance of *PIK3CA* status (range, 89%–94%).**

Conclusion. **Though discordance of *KRAS* status does occur, it is uncommon. When considering the downsides of testing metastatic tissue in all patients along with the low incidence of discordance, we conclude that that testing the primary tumor (or whatever tissue available) is sufficient for clinical decision making on EGFR inhibitor therapy.** ***The Oncologist*** 2011;16:1239–1249

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a member of the human epidermal growth factor receptor family of receptor tyrosine kinases, and has become an important target for anticancer therapy for a variety of solid tumors, including colorectal carcinoma (CRC), breast cancer, non-small cell lung cancer, and squamous cell carcinoma in the head and neck region [1]. After binding of ligand, the EGFR

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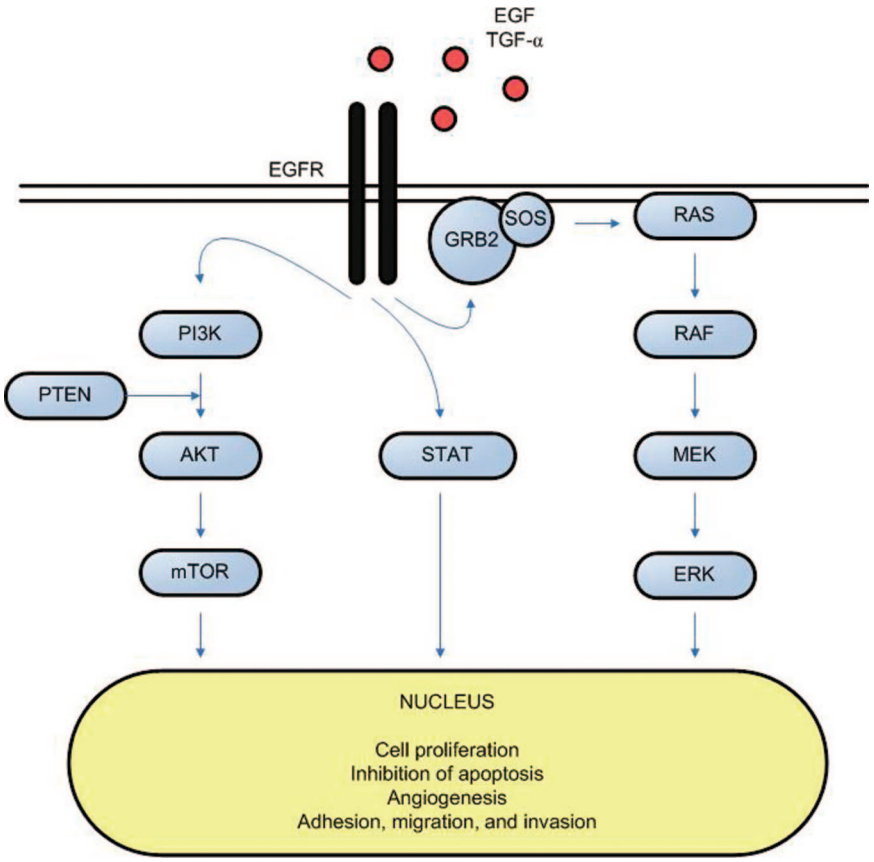

Figure 1. EGFR downstream pathways.

Abbreviations: AKT, protein kinase B; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-related kinase; GRB2, growth factor bound protein 2; MEK, mitogen-activated protein kinase–ERK kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted on chromosome ten; SOS, son of sevenless; STAT, signal transducer and activator of transcription; TGF-α, tumor growth factor α.

activates downstream pathways, leading to stimulation of angiogenesis, cell proliferation, migration, adhesion, invasion, and inhibition of apoptosis (Fig. 1) [2]. The considered main mechanism of action of the currently registered monoclonal antibodies directed against the EGFR, panitumumab and cetuximab, is their binding to the EGFR, thereby preventing ligand binding, thus preventing activation of the downstream signaling cascade, that is, the RAS/RAF/mitogen-activated protein kinase/extracellular signal–related kinase kinase/extracellular signal–related kinase, phosphatidylinositol 3-kinase (PI3K)/protein kinase B/mammalian target of rapamycin, and signal transducer and activator of transcription pathways [3].

Initial studies of panitumumab and cetuximab in CRC patients showed that these agents are effective in only a minority of patients [4, 5], leading to the need for biomarkers to enhance upfront patient selection. Much attention has been given to Kirsten rat sarcoma 2 viral oncogene homolog (*KRAS*) mutational status as a predictive marker, because activating mutations lead to a permanently active KRAS protein independent of EGFR activation [6]. *KRAS* mutations occur in 40% of all CRC patients [6–8]. The importance of the mutational status of *KRAS* was ultimately proven in post hoc analyses of the cetuximab and panitumumab pivotal trials, which showed a lack of response to EGFR inhibitors in patients harboring a mutation in codon 12 or 13 of *KRAS* [7, 8]. Notably, it was recently suggested that patients harboring a specific mutation in codon 13 (c.38G>A,p.G13D) possibly do benefit from EGFR inhibitor therapy [9]. Nonetheless, currently, EGFR monoclonal antibody therapy is indicated only in CRC patients (failing 5-FU, oxaliplatin, and irinotecan containing regimens) harboring wild-type (WT) *KRAS*.

However, even in patients with WT *KRAS* tumors, third-line responses are limited, with a rate of 17% for panitumumab and 12.8% for cetuximab monotherapy [7, 8]. Because treatment with these agents is associated with potential (severe) toxicity and high costs, it is important to find additional markers predictive of efficacy and to critically review all aspects of *KRAS* testing.

KRAS testing in clinical practice usually includes only mutations in codon 12 and 13 [7, 8, 10–15], because these account for, respectively, 70% and 20% of all activating *KRAS* mutations [16]. Analysis of mutations in codon 61 can be considered [10]; however, one has to realize that mutations in codon 61 account for ~5% of all activating *KRAS* mutations [17, 18] and can therefore only partly explain the limited response rates in patients free from mutations in codon 12 and 13. It has also been suggested that mutations in codon 146 are of relevance in selecting patients for cetuximab or panitumumab therapy [19, 20]; however, a recent study by de Roock et al. [16] showed that mutations in codon 146 are not associated with a lack of response to cetuximab.

Other genes involved in EGFR downstream pathways—like the v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), *PIK3CA* (encoding a subunit of the PI3K protein), and phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) [21]—have also been studied in order to find additional biomarkers to select patients that are most likely to benefit from panitumumab or cetuximab. *BRAF* mutations occur in ~8% of CRC patients and are thought to correlate with poor prognosis [22–24]. *KRAS* and *BRAF* mutations are considered mutually exclusive [24]. Most studies showed a lack of response to EGFR inhibitors in mutant *BRAF* patients [16, 22, 23, 25–28]. Loss of *PTEN* and *PIK3CA* mutations (in codon 9 and 20) are reported in, respectively, 30%–50% and 10%–30% of CRC patients [29], and both are considered to be potential predictive markers. Previous studies on loss of PTEN expression suggest a potential role as a predictive marker for response to EGFR inhibitors, but further studies are needed [30, 31]. Data on the importance of *PIK3CA* are conflicting; some studies show that *PIK3CA* mutations are associated with a lack of response to EGFR inhibitors [16, 31, 32], whereas others do not show such a relationship [13, 23]. Additionally, a recent study suggested that only mutations in codon 20 are of predictive value [16]. However, because there is no consensus yet on the importance of *BRAF* and *PIK3CA* mutational status and of loss of *PTEN*, these markers are not yet used in routine clinical decision making.

In addition, it is important to know what the concordance is between *KRAS* mutational status of the primary tumor and of metastases. In cases of discordance, it could be possible that patients who are thought to have mutant type (MT) *KRAS* tumors will not receive panitumumab- or cetuximab-based therapy, although their metastases are WT *KRAS* and thus they may benefit such therapy, or vice versa. Mutational analysis is usually performed on tissue of either the primary tumor or a metastasis, and although no advice is available on what technique to use, recommendations on testing techniques are made in the European Quality Assurance program (available at http://kras.eqascheme.org/) and the NCCN guidelines (available at http://www.nccn.org/index.asp). Because *KRAS* mutations are considered to be an early step in colorectal tumorigenesis [33], it is assumed that concordance between the primary tumor and metastases will be close to 100%. However, it has previously been mentioned that it is questionable whether the actual rate of concordance is indeed as high as assumed [21, 28]. Discordance between the primary tumor and metastases could possibly be explained by heterogeneity of the primary tumor, with progression of one specific clone as a result of selection, by technical issues, or by late acquirement or loss of *KRAS* mutations during disease progression.

This review gives an overview of studies testing concordance of mutational status between primary tumors and metastases in CRC patients, in particular, for those with *KRAS* mutations, but also for *BRAF*, *PIK3CA*, and of loss of *PTEN*, in order to make a recommendation on the use of mutational analysis in the clinic.

METHODS

A literature search within the PubMed database was conducted (on April 4, 2011) using the following keywords and combinations: *KRAS*, *BRAF*, *PTEN*, *PI3K*, *PIK3CA*, colorectal cancer, heterogeneity, *KRAS* mutation testing, *KRAS* testing, and *KRAS* assessment. References of all included articles were screened and included in this review when relevant. Additionally, a search within the American Society of Clinical Oncology (ASCO) abstracts database was conducted using the previously mentioned terms. Relevant abstracts from ASCO Annual Meetings and ASCO Gastrointestinal Cancers Symposium for the years 2000–2010 are included in this review.

Eligible abstracts or articles had to report concordance of mutational status of *KRAS*, *PIK3CA*, *BRAF*, or of loss of *PTEN*, or a combination of these mutations in primary tumors and matched metastases in CRC patients. Concordance of mutational status was defined as either the absence or presence of the mutation in both the primary tumor and the matched metastasis. Studies reporting concomitant *KRAS* and *BRAF* mutations in samples of either primary tumors or a metastasis were excluded because these mutations are considered to be mutually exclusive and the results reported in those studies may therefore be less reliable [24].

RESULTS

KRAS

Twenty-six possibly relevant studies were identified. Five studies were excluded, three because they tested *KRAS* sta-

Table 1. Studies on concordance of *KRAS* status in primary CRC tumors and matched metastases

Study	*n*	Frequency of *KRAS* mutation in primary tumor	Site of metastasis	Overall concordance	*KRAS* testing method
Knijn et al. (2011) [48]	305	35%	Liver, 100%	96%	Sequencing
Melucci et al. (2010) [49]	62	37%	Not specified	94%	Sequencing
Italiano et al. (2010) [47]	59	39%	Not specified	95%	Sequencing
Baldus et al. (2010) [54]	75	41%	Lymph node, 73%; other (27%)	76%	Sequencing and pyrosequencing
Cejas et al. (2009) [45]	110	34%	Liver, 83%; lung, 17%	94%	Sequencing
Molinari et al. (2009) [28]	37	43%	Liver, 74%; other, 26%; lymph node, 41%[a]	92%	Sequencing
Loupakis et al. (2009) [30]	43	40%	Not specified	95%	Sequencing
Garm Spindler et al. (2009) [46]	31	29%	Not specified	94%	Sequencing
Santini et al. (2008) [50]	99	38%	Liver, 81%; lung, 7%; other, 12%	96%	Sequencing
Artale et al. (2008) [44]	48	27%	Liver, 81%; other, 19%	94%	Sequencing
Etienne-Grimaldi et al. (2008) [39]	48	33%	Liver, 100%	100%	PCR-RFLP
Perrone et al. (2009) [31]	10	20%	Not specified	80%	Sequencing
Albanese et al. (2004) [53]	30	47%	Liver, 100%	70%	SSCP
Zauber et al. (2003) [43]	42	52%	Lymph node, 93%; liver, 5%; mesentery, 2%	100%	SSCP
Thebo at al. (2000) [55]	20	100%	Lymph node, 100%	80%	AS-PCR
Schimanski et al. (1999) [51]	22	95%	Liver, 100%	95%	PCR-RFLP
Al-Mulla et al. (1998) [52]	47	34%	Lymph node, NR; liver, NR	83%	ASO
Finkelstein et al. (1993) [40]	NR	35%	Not specified	100%	Sequencing
Losi et al. (1992) [41]	18	83%	Liver, 33%; other, 67%	100%	AS-PCR
Suchy et al. (1992) [42]	66	21%	Not specified	100%	ASO
Oudejans et al. (1991) [56]	31	42%	Lung, liver	87%	ASO

[a]Molinari et al. [28] tested the *KRAS* status of both matched metastases and lymph nodes in 15 cases.
Abbreviations: AS-PCR, allele-specific polymerase chain reaction; ASO, allele-specific oligonucleotide hybridization; CRC, colorectal cancer; *KRAS*, Kirsten rat sarcoma 2 viral oncogene homolog; MT = mutant type *KRAS*; NR, not reported; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; WT = wild type *KRAS*.

tus to find possible micrometastases [34–36] and the fourth because it reported concomitant *KRAS* and *BRAF* mutations in 10 of 28 patients [37]. The fifth study, testing concordance of >20 different mutations in 10 patients, was excluded because it did not report the concordance of *KRAS* [38]. In total, 21 studies testing the concordance of *KRAS* mutational status were included in this review (Table 1). Five studies found a concordance rate of 100% [39–43], and in 10 additional studies the concordance rate was ≥90% [28, 30, 44–51]. Five of the 21 studies reported concordance rates <85% [31, 52–55], with 76% being the lowest rate reported [54]. The overall concordance rate of the studies reported in Table 1 is 93%. The study by Finkelstein et al. [40] was excluded in this calculation because it did not report the number of patients.

The tissue analyzed was mostly formalin fixed and paraffin embedded, although Schimanski et al. [51] and Etienne-Grimaldi et al. [39] used frozen material instead, and two other studies did not report whether they used fixed or frozen material [44, 49]. DNA was isolated using various techniques, although the majority used the QIAmp DNA kit (Qiagen, Germantown, MD). Mutational analysis was performed by polymerase chain reaction (PCR) followed by sequencing in 12 studies (Table 1). Other methods used for *KRAS* analysis were PCR–reaction fragment length polymorphism, allele-specific PCR (AS-PCR), allele-specific

Table 2. Concordance of *KRAS* status in the primary tumor and hepatic versus extrahepatic metastases

Study	*n*	Site of metastasis	Concordance, hepatic metastases	Concordance, extrahepatic metastases[a]	Concordance, lymph node metastases
Knijn et al. (2011) [48]	305	Liver, 100%	96%	NE	NE
Baldus et al. (2010) [54]	75	Lymph node, 73%; other, 27%	NR	NR	68%
Cejas et al. (2009) [45]	110	Liver, 85%; lung, 15%	95%	88%	NE
Molinari et al. (2009) [28]	37	Liver, 76%; other, 24%; lymph node, 41%[b]	NR	NE	100%
Santini et al. (2008) [50]	99	Liver, 81%; other, 19%	96%	95%	NE
Artale et al. (2008) [44]	48	Liver, 81%; other, 19%	97%	78%	NR
Etienne-Grimaldi et al. (2008) [39]	48	Liver, 100%	100%	NE	NE
Albanese et al. (2004) [53]	30	Liver, 100%	70%	NE	NE
Zauber et al. (2003) [43]	42	Lymph node, 93%; liver, 5%; mesentery, 2%	100%	100%	100%
Thebo et al. (2000) [55]	20	Lymph node, 100%	NE	80%	80%
Schimanski et al. (1999) [51]	22	Liver, 100%	95%	NE	NE
Losi et al. (1992) [41]	18	Liver, 33%; other, 67%	100%	100%	NE
Total	854	Hepatic, 73%; extrahepatic, 22%[c,d]; lymph node, 15%	94%	86%[a]	84%

[a]Including lymph node metastases when available.
[b]Molinari et al. [28] tested *KRAS* status of both matched metastases and lymph nodes in 15 cases.
[c]Including lymph node metastases.
[d]Sum of percentages is not 100% because some studies did not report concordance rates for all individual metastatic sites.
Abbreviations: *KRAS*, Kirsten rat sarcoma 2 viral oncogene homolog; NE, not evaluated; NR, not reported.

oligonucleotide hybridization (ASO), single-strand conformation polymorphism, and pyrosequencing. All studies tested for mutations in codon 12 and 13, except for Oudejans et al. [56], who also tested for mutations in codon 61, and Suchy et al. [42], who tested only for mutations in codon 12. Most authors reported codon 12 and 13 mutations in a ratio as would be expected according to the literature (i.e., approximately 80% in codon 12 and 20% in codon 13 when testing only codon 12 and 13); however, two reported relatively more codon 13 mutations [53, 55] and two reported codon 13 mutations in $<10\%$ of cases [43, 56].

Concordance of KRAS *in Hepatic Versus Extrahepatic Metastases*

A subanalysis was done with regard to concordance of *KRAS* in primary tumors and hepatic versus extrahepatic metastases, in order to study whether the localization of a metastasis predicts the risk for discordance. Because the lowest concordance rates were reported by studies testing lymph node metastases, concordance in lymph node metastases was also analyzed separately. When combining all studies that defined the site of metastasis, we found a concordance rate of 95% for hepatic and 86% for extrahepatic metastases (including lymph node metastases) (p-value = .01 by χ^2 test), suggesting that there is a difference in concordance between the primary tumor and hepatic versus extrahepatic metastases (Table 2). When comparing concordance of hepatic metastases with concordance of lymph node metastases (95% versus 84%; p-value $< .01$ by χ^2 test), it seems that discordance with the primary tumor occurs more frequently in lymph node metastases.

Concordance of KRAS *in Patients with Primary WT Versus Primary MT Tumors*

An additional subanalysis was done with regard to concordance in patients with primary WT versus primary MT tumors, in order to observe whether the mutational status of the primary tumor predicts the presence of discordance. When selecting studies that defined the status of both tissues, in cases of discordance, it was found that discordance occurred in 14% of patients harboring a *KRAS* mutation, compared with 5% of patients with WT *KRAS* tumors (95% versus 86%; p-value $< .01$ by χ^2 test) (Table 3).

BRAF

Seven studies on concordance of *BRAF* status were found (Table 4) [28, 31, 44, 47, 54, 57, 58], and all tested *BRAF* status by sequencing. Baldus et al. [54] additionally performed pyrosequencing. All tested for mutations in exon 15 (or only the classical V600E mutations), and only Perrone

Table 3. Concordance of *KRAS* status in WT primary versus MT primary patients

Study	*n*	Overall concordance	Concordance in WT primary patients	Concordance in MT primary patients
Knijn et al. (2011) [48]	305	96%	99%	91%
Italiano et al. (2010) [47]	59	95%	94%	96%
Baldus et al. (2010) [54]	75	76%	94%	59%
Cejas et al. (2009) [68]	110	94%	93%	95%
Molinari et al. (2009) [28]	37	92%	95%	88%
Loupakis et al. (2009) [30]	43	95%	92%	100%
Garm Spindler et al. (2009) [46]	31	94%	100%	82%
Santini et al. (2008) [50]	99	96%	98%	92%
Artale et al. (2008) [44]	48	94%	95%	91%
Etienne-Grimaldi et al. (2008) [39]	48	100%	100%	100%
Perrone et al. (2009) [31]	10	80%	88%	50%
Albanese et al. (2004) [53]	30	70%	75%	64%
Zauber et al. (2003) [43]	42	100%	100%	100%
Thebo et al. (2000) [55]	20	80%	NE	80%
Schimanski et al. (1999) [51]	22	95%	0%	100%
Al-Mulla et al. (1998) [52]	47	83%	81%	88%
Losi et al. (1992) [41]	18	100%	100%	100%
Oudejans et al. (1991) [56]	31	87%	84%	92%
Total	1,075	92%	95%	86%

Abbreviations: *KRAS*, Kirsten rat sarcoma 2 viral oncogene homolog; MT, mutant type *KRAS*; NE, not evaluated; WT, wild-type *KRAS*.

Table 4. Studies on concordance of *BRAF* in primary CRC tumors and metastases

Study	*n*	*BRAF* mutation in primary tumor (*n*)	Overall concordance	Concordance in WT primary patients	Concordance in MT primary patients	*BRAF* testing method
Santini et al. (2010) [58]	203	6% (13)	97%	99%	62%	NR
Cejas et al. (2010) [57]	117	NR	100%	100%	100%	NR
Italiano et al. (2010) [47]	48	3% (1)	98%	98%	100%	Sequencing
Baldus et al. (2010) [54]	75	7% (5)	97%	100%	83%[a]	Sequencing
Molinari et al. (2009) [28]	36	6% (2)	100%	100%	100%	Sequencing
Artale et al. (2008) [44]	48	4% (2)	98%	100%	50%	Sequencing
Perrone et al. (2009) [31]	11	0% (0)	91%	91%	NE	Sequencing

[a]Baldus et al. [54] initially found discordant mutational status in 2 patients; however, analysis of additional lymph nodes showed concordant results in 1 of those 2 patients.
Abbreviations: *BRAF*, v-raf murine sarcoma viral oncogene homolog B1; CRC, colorectal cancer; MT, mutant type *BRAF*; NE, not evaluated; NR, not reported; WT, wild-type *BRAF*.

et al. [31] additionally tested codon 11. Because of the low prevalence of *BRAF* mutations in general, the number of patients with MT *BRAF* tumors in these studies was small (range, 0–13). The overall concordance of the studies on concordance of *BRAF* was 98%, and the overall concordance in patients with a *BRAF* mutation in their primary tumor was 70%. Santini et al. [58] reported discordant mutational status in five of 13 patients with a *BRAF* mutation in their primary tumor; however, the overall concordance rate in their analysis of 203 patients was 98%. Baldus et al. [54] found concordant results in two patients; however, when testing additional lymph nodes of both patients, results were eventually discordant in only one patient.

Table 5. Studies on concordance of loss of *PTEN* in primary CRC tumors and metastases

Study	*n*	Loss of *PTEN* in primary tumor (*n*)	Concordance	*PTEN* testing method
Cejas et al. (2010) [57]	117	NR	73%	NR
Sood et al. (2010) [60]	51	59% (30)	47%	IHC
Negri et al. (2009) [59]	20	NR	75%	FISH
Molinari et al. (2009) [28]	38	21% (8)	89%	IHC
Loupakis et al. (2009) [30]	45	NR	60%	IHC
Perrone et al. (2009) [31]	8	NR	63%	FISH

Abbreviations: CRC, colorectal cancer; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NR, not reported; PTEN, phosphatase and tensin homologue deleted on chromosome ten.

PTEN

Six studies on concordance of loss of *PTEN* were found (Table 5), and the range of concordance was 47%–89% [28, 30, 31, 57, 59, 60]. The overall concordance of the studies reported in Table 5 is 68%. Evaluation of *PTEN* was mainly performed by immunohistochemistry or fluorescence in situ hybridization. Perrone et al. [31] additionally tested *PTEN* mutational status, and reported a concordance rate of 82%.

PIK3CA

Three studies on concordance of *PIK3CA* status were found. Perrone et al. [31] reported discordance of *PIK3CA* status in one of 11 patients. Concordance was therefore 91%. Baldus et al. [54] found a discordant mutational status in eight of 75 patients; concordance in that study was therefore 89%. Cejas et al. [57] tested concordance of *PIK3CA* status in WT *KRAS* patients (number of patients not reported) and found a concordant mutational status in 94% of cases. Perrone et al. [31] and Baldus et al. [54] tested the hotspots exon 9 and 20 for *PIK3CA* mutations using sequencing; the abstract by Cejas et al. [57] did not report what exon was tested nor did it report the technique they used to do so. Perrone et al. [31] performed all analyses twice and Baldus et al. [54] performed an additional analysis using pyrosequencing.

Discussion

Currently, *KRAS* mutational status is the only predictive marker used in clinical practice in deciding whether or not to start EGFR inhibitor therapy. Although concordance of *KRAS* status between primary tumors and metastases has always been considered to be close to perfect, cases of discordance have been reported in the literature. Therefore, we raised the question of whether or not *KRAS* mutational analysis should be performed on metastatic tissue and whether or not it is necessary to obtain tissue of a metastasis if not yet available. Here, we summarize all studies on concordance of *KRAS* reported so far, and show that although discordance of mutational status does occur, it is extremely rare. Although this retrospective study does not include new information, and discusses various nonidentical studies, the results presented here suggest that testing on whatever tissue available is sufficient in clinical decision making on whether or not to start EGFR inhibitor therapy, especially when considering the burden and potential risks for patients needing to undergo additional biopsies to obtain metastatic tissue. Moreover, in cases of discordance, it is highly questionable whether or not the mutational status of one metastasis is representative of the mutational status of other metastases.

To our knowledge, this review summarizes the largest series of studies testing concordance of *KRAS* status in primary tumors and matched metastases, and it shows that concordance of *KRAS* status is generally >90%. The main question is whether or not these rates result in the need to test the *KRAS* status of metastases prior to treatment with EGFR inhibitors. Nevertheless, although previously stated to be impossible, this shows that discordance of *KRAS* mutational status does occur. Possible explanations according to the literature are acquirement of mutations during or after metastasizing, false-negative and false-positive test results, and heterogeneity of CRC tumors. Late occurrence of mutations, as suggested by Albanese et al. [53] and Zauber et al. [43], is a questionable explanation because *KRAS* mutations have been proven to be an early step in tumorigenesis [33, 61, 62]. False-negative and false-positive test results could also be an explanation for discordance. The technique most widely used for *KRAS* testing—in general and in this review—is sequencing. This technique is known to be highly specific; however, its sensitivity greatly depends on the number of tumor cells present in the tissue sample [63]. This might be relevant in patients who have already received chemotherapy, because tumor cells may be sparse in

these cases. However, four of the 12 studies using sequencing mentioned that the samples tested in their study had to contain ≥70% tumor cells [28, 45, 47, 54]. All four of them reported discordance in some cases. As for the reliability of the other techniques used in the studies reported in Table 1, a paper by Oliner et al. [64], evaluating five different *KRAS* tests, showed that classic DNA sequencing as well as pyrosequencing and AS-PCR provide accurate *KRAS* analysis. The commercial ASO kit described in that paper was reported to be less accurate. The concordance rates found in studies using the latter method may therefore be less reliable and lower than those from studies using other techniques. However, only a minority of the studies reported in this review used techniques other than sequencing. Additionally, most studies on concordance have carefully reviewed the quality and tumor content of the samples included in their analysis, and some studies performed sequencing twice, starting from different amplification reactions [28, 31, 53], whereas others had two independent observers to determine *KRAS* status [50, 52], or re-evaluated their results using pyrosequencing [54]. Therefore, although it is possible that a small number of the discordant cases are a result of poor quality testing techniques, false test results are not likely to explain the discordant cases in the majority of these studies.

Most likely, discordance is caused by heterogeneity of the primary tumor followed by the progression of one clone, resulting in metastases with the mutational pattern of that specific clone. CRC was originally considered to have a homogeneous mutational profile [33, 65]. However, although a homogeneous profile is found in the majority of CRC tissue, cases of heterogeneity of *KRAS* status within one tumor have been reported [16, 52, 66, 67]. Ishii et al. [67] searched for heterogeneity of *KRAS* status in CRC by examining multiple samples from the primary tumor of 21 CRC patients. Heterogeneity in *KRAS* status was found in seven of 21 patients. Baldus et al. [54] performed analysis on different sections—from the tumor center and from the invasion front of the primary tumor—and on different lymph nodes of patients with discordant mutational status. A heterogeneous pattern was found in eight of 41 patients, and because the prevalence of *KRAS* mutations was highest in the samples from the tumor center, the authors suggested that *KRAS* analysis should preferably be done on samples from the center of a tumor. Apparently heterogeneity of *KRAS* status in CRC does occur.

Few studies on the mutational heterogeneity of biomarkers other than *KRAS* have been published so far. Concordance of *BRAF* status seems to be comparable with concordance of *KRAS* status. However, the prevalence of *BRAF* mutations is low, and the studies discussed in this review contained only a few patients with *BRAF* mutations; therefore, it is difficult to reliably determine the rate of discordance of *BRAF* mutational status. Nonetheless, most patients with discordant results carried a *BRAF* mutation in their primary tumor. Currently, *BRAF* mutational status is not (yet) used in the clinical decision making on whether or not to start cetuximab- or panitumumab-based therapy. However, if *BRAF* analysis were to become part of standard care, one could consider additionally testing the mutational status of metastases of patients harboring a *BRAF* mutation in their primary tumor.

Lower concordance rates were reported for loss of *PTEN*. However, it is important to emphasize the fact that no standardized method is currently available for *PTEN* analysis [16]. Additionally, because the role of *PTEN* in predicting response to EGFR inhibitors is still unclear, both the actual rate and the clinical relevance of discordance of this possible biomarker also remain unknown. Discordance in loss of *PTEN* and in the mutational status of *PIK3CA* and *BRAF* is also likely caused by heterogeneity of the primary tumor.

Detecting the presence of discordance in the *KRAS* status of CRC patients, one could wonder whether the mutational status of the primary tumor predicts the likelihood of discordance. A subanalysis was performed to check concordance rates in WT primary and MT primary patients, and it showed a small difference, with lower concordance rates in patients with MT primary tumors (95% versus 86%; p-value $< .01$). Additionally, it has also been suggested that concordance rates are related to the location of metastases [44, 54]. Therefore, an additional subanalysis was performed to evaluate concordance of primary tumors and hepatic versus extrahepatic metastases and versus lymph node metastases, which showed that concordance rates are indeed related to hepatic or extrahepatic localization of metastases. Additionally, it showed that the *KRAS* status of lymph nodes is the least concordant with the mutational status of the primary tumor. Unfortunately, mutational status of distant metastases was not documented in these specific cases, so it is unclear whether the mutational status of lymph nodes is representative of the mutational status of distant metastases.

Overall, the literature reported so far shows that discordance of *KRAS* status between primary CRC and metastases is found in <10% of all CRC patients. The main question is whether this rate results in the need to (additionally) test the mutational status of a metastasis. When trying to answer this question, one should consider the following issues. First, when no tissue from metastases is available, patients will need to undergo an additional biopsy to obtain the required tissue, possibly leading to complications such as

bleeding or infection, along with the burden of undergoing an extra procedure. Second, a double *KRAS* analysis for each patient will significantly increase costs. Moreover, when a metastasis indeed shows a different mutational status, there is no guarantee that the status of this metastasis is representative of the mutational status of other metastases. Additionally, as was shown in this review, the *KRAS* mutational status of the primary tumor is representative of that of metastases in >90% of all patients. However, it is important to emphasize that the results presented in this review are based on various studies of various sizes, using various methods of *KRAS* analysis. Future studies examining large series, such as the one published by Knijn et al. [48], and testing not only *KRAS* but also *BRAF*, *PTEN*, and *PIKCA* would make it possible to draw definitive conclusions on the concordance rates of these markers. Furthermore, a large series of paired samples exploring the concordance rates of these and various other genes (like *NRAS*, *P53*, and *EGFR*) would provide valuable insight into the carcinogenesis and metastasizing patterns of CRC and could possibly guide treatment options for CRC patients. Nonetheless, considering the above-mentioned issues, we conclude that, based on the currently available literature, additionally testing metastatic tissue is currently not justified, and that testing *KRAS* mutational status of the primary tissue (or whatever tissue available) is sufficient in clinical decision making on the initiation of EGFR inhibitor therapy.

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